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Human Immunodeficiency Virus-related Microbial Translocation and Progression of Hepatitis C:

HIV, HCV, Microbial Translocation and Liver Disease

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Abstract

Background & Aims—HIV-1 infection has been associated with enhanced microbial translocation, and microbial translocation is a mechanism through which alcohol and some enteric conditions cause liver disease. We hypothesized that HIV promotes liver disease by enhancing microbial translocation.

Methods—We studied human cohorts in which hepatitis C virus (HCV) and HIV outcomes were carefully characterized.

Results—HIV-related CD4+ lymphocyte depletion was strongly associated with microbial translocation as indicated by elevated levels of circulating lipopolysaccharide (LPS), LPS binding protein, soluble CD14, fucose-binding lectin (AAL) reactive to IgG specific for the alpha galactose epitope, and suppressed levels of endotoxin-core antibodies (EndoCAb IgM) in HIV-infected subjects compared with the same persons before they had HIV infection and compared with HIV-uninfected subjects. The same measures of microbial translocation were strongly associated with HCV-related liver disease progression (cirrhosis), e.g. LPS, odds ratio 19.0 ($p = 0.002$), AAL, odds ratio 27.8 ($p < 0.0001$); in addition, levels of LPS were elevated prior to recognition of cirrhosis.

Conclusions—Microbial translocation may be a fundamental mechanism through which HIV accelerates progression of chronic liver disease.

INTRODUCTION

In Europe, the United States and Australia, liver disease has emerged as a leading cause of death among HIV-infected persons and most is due to chronic viral hepatitis.¹ Liver disease

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burden is increased because HIV-infected persons are at increased risk of chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and HIV-related immunosuppression accelerates liver disease progression.^{2, 3} However, the mechanisms through which HIV infection increases the risk of liver disease are unknown.

HIV infection causes CD4+ lymphocyte depletion that occurs in gastrointestinal tissues within the first months of infection.⁴⁻⁹ HIV-related depletion of mucosal CD4+ lymphocytes has been linked with disruption of gut epithelial integrity and increased mucosal translocation of bacteria and bacterial products including lipopolysaccharide (LPS)¹⁰, the inflammatory component of the Gram-negative bacteria cell wall. Recently, the magnitude of microbial translocation, as reflected by blood levels of LPS and host components required for its binding and recognition by macrophages, was strongly correlated with HIV-related immune activation, eventual CD4+ lymphocyte depletion in peripheral blood, and clinical expression of disease.¹⁰ Additionally, naturally occurring LPS-binding immunoglobulin (EndoCAb IgM) was found to have an inverse relationship with LPS.

Hepatic tissues, and in particular, liver macrophages (Kupffer cells) are directly affected by microbial translocation. Free LPS binds to Kupffer cells via interactions with circulating LPS binding protein (LBP) and CD14. The membrane-bound LPS inflammatory complex signals via Toll-like receptor 4 (TLR4) and the transcription factor NFκB, which upregulates proinflammatory and profibrogenic cytokines such as tumor necrosis factor (TNF)α, IL-1, IL-6, and IL-12.^{11, 12}

Alcohol-induced liver disease has been linked to microbial translocation.^{13, 14} In several animal studies, alcohol use has been associated with increased enteric microbial burden and translocation, resulting in increased markers of microbial translocation, including LPS.^{14, 15} In animal models, both sensitization and tolerance of Kupffer cells has been described and alcohol related liver disease can be reduced by suppression of microbial burden with antibiotics or inhibition of effector cytokines such as TNFα.¹⁵ Recently, it was shown that TLR4 activation by LPS upregulates chemokine secretion and sensitizes stellate cells to transforming growth factor β and the activating effects of Kupffer cells.¹⁶ Microbial translocation has also been implicated in liver disease associated with other enteric processes, such as graft versus host disease and celiac sprue.¹⁷⁻²⁰

We hypothesized that, like alcohol, HIV might accelerate liver disease through microbial translocation caused by CD4+ lymphocyte depletion and immune activation, especially in a context like chronic HCV infection in which there is chronic hepatic inflammation. To test the hypothesis, we studied cohorts of human subjects before and after HIV and HCV infections, as HIV-related CD4+ lymphocyte depletion occurred, and according to carefully-defined liver disease outcomes.

METHODS

Study Population

For this investigation, subjects were chosen from three distinct ongoing cohorts in which liver disease, HIV infection, and HCV infection were carefully evaluated and serum specimens were archived at -80 °C. All subjects provided informed consent for testing through a protocol approved by the Committees on Human Research of the Johns Hopkins School of Medicine or Bloomberg School of Public Health.

Prevalent liver disease group—We identified the first 100 consecutive samples with paired liver biopsies for testing, then excluded those with unclear liver disease status. Cases had either clinically-manifest end stage liver disease (defined as ascites, esophageal varices,

or hepatic encephalopathy) or liver-biopsy-proven cirrhosis (metavir 3–4) and controls had at least two liver biopsies 3–5 years apart with no more than minimal fibrosis (metavir 0–1). Of the seventy-one controls, all had at least two liver staging determinations during the study interval. For all but two subjects this included two biopsies, while two subjects had a biopsy and a fibroscan. All results at two time points were consistent with a Metavir 0/1 stage at the most recent determination, except for five subjects who had a Metavir 2 stage on the second biopsy. These subjects derived from a community based study of HCV progression among injection drug users (The AIDS Link to Intravenous Experience [ALIVE] Cohort), as previously described.^{21–23} Alcohol use in the six months prior to outcome ascertainment was measured as a combined variable representing no use, less than one drink per day, and greater than or equal to one drink per day.

HIV seroconversion cohort—Within the ALIVE cohort, subjects were screened for HIV infection every six months since 1988.²⁴ By March of 2002, 309 persons had acquired HIV-1 infection. Twenty-nine of these subjects with chronic hepatitis C were studied because there were already detailed studies of HIV RNA, CD4+ lymphocyte trajectories and HCV humoral immune responses and serum samples were available before HIV infection (2.8 to 26.4 months before first antibody positive visit) and at two intervals after HIV infection (“first post”: 7.7–88.8 months after first positive and “second post” 16.6–141.1 months after first positive).²⁵

HCV seroconversion cohort—HCV-uninfected injection drug users are followed monthly in the Risk Evaluation Assessment of Community Health (REACH) Cohort, as previously described.²⁶ From 1997 to 2002, a total of 179 subjects were enrolled, and 62 (34.6%) had HCV seroconversion. From these 34 were studied because serum was available at defined time intervals before and after HCV seroconversion.

Microbial translocation and immune activation assays

To inactivate plasma proteins, plasma and serum samples were diluted to 20% in endotoxin-free water and heated to 80°C for 12 minutes. LPS levels were measured using a Limulus Amebocyte Lysate assay (LONZA, Walkersville, MD, USA) according to the manufacturer’s protocol. To avoid sample interference with substrate absorbance, p-nitroaniline was derivatized via the addition of diazo-coupling reagents.²⁷ Samples were run in duplicate and background, if present, was subtracted. Commercially available ELISA kits were used to measure plasma and serum levels of LBP, EndoCAB IgM (Cell Sciences, Canton, MA, USA) and soluble CD14 (sCD14, R&D Systems, Minneapolis, MN, USA). **Lectin Fluorophore-Linked Immunosorbent Assay (FLISA)**: the amount of an altered IgG, itself specific for a heterophilic (alpha-galactose) epitope, has previously been strongly associated with stage of liver disease.²⁸ The altered (agalactosylated) antibody has an exposed fucose residue, which allows it to be detected by a fucose-binding lectin derived from *Aleuria aurantia* (AAL). An AAL-based FLISA was used to quantify the amount of this altered immunoglobulin.

Statistical analysis

The distributions of microbial translocation marker tests were examined and log₁₀ transformed to normalize. Values of LPS <10 pg/mL were below the linear range and were all assigned a value of 5 pg/mL before transformation. Linear regression was used to model microbial translocation markers. Wilcoxon-rank sum tests were used to compare microbial translocation markers between HIV-stratified groups. Logistic regression was used to examine the relationship between the binary liver outcome and the covariates. A Generalized Estimator Equation (G.E.E.) was used to compare longitudinally-obtained HIV seroconversion data and markers of microbial translocation. In all models CD4+ lymphocyte count was included to assess the association of depletion with change in marker level, adjusting for time and that person’s pre-HIV baseline. Shown is a model of the final microbial translocation marker,

adjusting for time and pre-seroconversion value. Also computed (and showing similar findings) were models of the change of marker values divided by time and the difference in the last compared to the first, adjusted for time. Covariates with a p-value <0.05 in univariate analysis were entered into a multivariate model in a stepwise manner. A p-value of <0.05 was considered significant.

RESULTS

Prevalent liver disease group and microbial translocation markers

A total of 88 HCV-infected persons had either cirrhosis or end stage liver disease (17 cases) or clear evidence of minimal liver disease (71 controls). The mean age of subjects was 43.4 years at time of testing, 79.6 % were male, 96.6 % were African American, mean HCV RNA level was 12,928,360 IU/mL (7.1 log₁₀) and 31.8 % were HIV-infected (Table 1). The associations of microbial translocation markers with factors like age, gender, and alcohol use that might affect these markers and/or liver disease were examined by linear regression. Older age was associated with LPS (p=0.038), sCD14 (p=0.013), and AAL (p<0.0001). Similarly, female gender was associated with higher LPS (p=0.007), sCD14 (p=0.003), and AAL (p=0.007). Statistically significant associations were not detected between microbial translocation markers and race, or alcohol use (data not shown). Persons who acknowledged active injection drug use in the six month period before study had higher levels of EndoCAb IgM (p=0.0005).

HIV infection is associated with microbial translocation

Compared to 60 HIV-uninfected subjects, HIV-infected persons (n=28) had higher levels of LPS (p=0.011) and LBP (p=0.024), and lower EndoCAb IgM (p=0.018) when analyzed using linear regression. There was also a trend towards increased levels of sCD14 in HIV-infected persons (p=0.067). No difference was detected in AAL in HIV-infected versus uninfected persons overall. To evaluate the role of CD4+ lymphocyte depletion, data were further stratified. HIV-infected persons with CD4+ lymphocyte counts <350/mm³ had significant differences in LPS (p=0.028), sCD14 (p=0.038), and EndoCAb IgM (p=0.031) compared to persons without HIV (Figure 1).

To assess potential interactions between HIV, liver disease stage and microbial translocation, bivariate models of LPS were constructed. The odds of having elevated LPS were highest in the group with both HIV and cirrhosis. Individually, it appeared that each (HIV or liver disease) was associated with elevated LPS levels, but the association was much stronger for liver disease and that for HIV without liver disease was of marginal statistical significance (Table 2).

HIV seroconversion and microbial translocation

To assess the temporal association between microbial translocation and HIV infection, microbial translocation markers were compared in the same persons in serum collected before and at two points after HIV infection. After adjusting for the duration of follow-up and the pre-HIV infection value, subjects with CD4+ lymphocyte counts <200/mm³ had higher LPS (0.88 log increase, p=0.03) and higher sCD14 (0.1 log increase, p=0.0006) compared to persons whose CD4+ lymphocyte counts remain higher.

HCV infection itself does not cause increased microbial translocation

To assess if HCV infection itself causes microbial translocation, we evaluated markers in 34 persons with acute HCV infection (REACH cohort). When compared to pre-HCV infection levels, statistically significant increases were only detected in sCD14 in samples collected a

median (range) 3.7 (0.3 – 17.2) months before seroconversion and then a median of 19 (12.9 – 32.6) months after seroconversion (Figure 2).

Liver disease progression, HIV, and microbial translocation

In the 88 subjects of the prevalent disease cohort, we examined the association between HIV-infection and liver disease progression. Specifically, we were interested in the effect of CD4+ lymphocyte depletion. Compared to persons with preserved CD4+ lymphocyte counts, liver disease progression was detected 7.0-fold more often (95% CI 1.36 – 36.31, $p=0.02$) in HIV-infected persons with CD4+ lymphocyte count $<350/\text{mm}^3$.

When compared to subjects with undetectable or moderately elevated LPS, liver disease progression was detected 19.0-fold more often (95% CI 2.98 – 120.79, $p=0.0018$) in those who had levels in the upper quartile ($>42 \text{ pg/mL}$). Similar results were found for sCD14 and AAL (Table 3). Consistent with this finding AAL, a marker of liver disease in HCV-infected persons, was positively correlated with LPS ($r=0.24$, $p=0.03$) and sCD14 ($r=0.41$, $p<0.0001$) (data not shown). An inverse association was noted with EndoCAb IgM, consistent with the earlier association with HIV. The associations of HIV and microbial translocation with liver disease progression were maintained in a multivariate logistic regression model adjusting for age, CD4+ lymphocyte count, and alcohol exposure measured in the six months prior to outcome ascertainment.

Temporal association between microbial translocation and liver disease

A look back study was performed on 53 persons in the prevalent disease cohort who had at least 2 specimens collected at least 8 years before disease ascertainment. A total of 188 serum specimens collected a median (range) 4.4 (0.1 – 17.1) years before liver disease progression were examined. Compared to baseline (first sample tested), a statistically significant difference was seen in LPS levels up to 1 year before liver disease though not earlier ($p=0.001$, data not shown).

DISCUSSION

In this study, we confirmed that HIV-related CD4+ lymphocyte depletion was associated with microbial translocation and established a link between HIV-related microbial translocation and the severity of liver disease. These data suggest that microbial translocation may be a novel mechanism by which HIV accelerates liver disease.

The link between HIV and microbial translocation was already made by Brenchley et al.¹⁰ We confirm their cross sectional analyses and extend these observations by finding that microbial translocation is chiefly evident after CD4+ lymphocyte depletion and in individuals with cirrhosis. By studying persons before and after HIV seroconversion, we were able to account for differences between subjects in the ‘baseline’ levels of these markers and to examine the association of HIV infection before CD4+ lymphocyte depletion occurs. These data support the proposed role of microbial translocation in the pathogenesis of HIV-related disease.

Two subjects with CD4+ lymphocyte depletion were noted to have extremely high LPS levels and an additional two subjects had very high sCD14 levels. However, we do not believe that these outlier data disproportionately contribute to the results because non-parametric tests were used that assessed rank order rather than absolute value. In addition, we repeated the analyses by Winsorizing²⁹ and associations between LPS and HIV CD4+ lymphocyte depletion remained ($p=0.011 \rightarrow p=0.013$).

In this investigation, the risk of cirrhosis was 7.0 fold higher in persons with CD4+ lymphocyte depletion, a finding that is consistent with (or stronger than) what has been reported by other

investigators.^{2, 30, 31} Liver disease was also strongly associated with microbial translocation. While not proven, these findings are consistent with the hypothesis that microbial translocation contributes to both immune activation and progression of liver disease (Figure 3).

Microbial translocation has previously been associated with liver disease. For example, elevated LPS levels were associated with HCV-related liver disease by Caradonna et al., and LPS levels diminished in those who achieved interferon-related virus suppression.³² Additionally, in animal models alcohol has been linked to LPS and cirrhosis.^{15, 33–35} Interestingly, like HIV infection, alcohol use is strongly associated in epidemiologic studies with HCV-related liver disease progression.^{36–40} In fact, for each (alcohol or HIV-infection) there is a more than additive association with HCV-related liver disease, but the mechanism is not known.^{2, 36–39}

Microbial translocation is central to the pathogenesis of alcohol-related liver disease.^{13, 15, 33–35} Recent work in murine models suggests a model in which LPS activation of TLR4 increases stellate cell susceptibility to inflammatory stimuli.¹⁶ While not evaluated experimentally, this work provides a context in which chronic LPS stimulation might have more than an additive effect on the liver disease caused by a second process like chronic viral hepatitis. Interestingly, the effect of alcohol on liver disease progression can be abrogated by gut sterilization with antimicrobials.¹⁵ Recent literature in an acute liver injury model has further suggested that replacement of LPS producing bacteria with so-called probiotic flora diminishes acute liver injury.⁴¹ We are not aware of studies extending this work to chronic viral hepatitis. We did not find a confounding effect of alcohol in our study, though alcohol use remains a difficult variable to quantify in experimental human studies.

Of the markers studied, we found the strongest associations among liver disease, HIV-related CD4+ lymphocyte depletion, and the amount of AAL-reactive IgG specific for the alpha galactose epitope, which is a recently described biomarker closely correlated with liver fibrosis and cirrhosis.²⁸ The heterophilic alpha-galactose epitope is most notably a major antigen on Gram-negative micro-organisms. Thus, although the natural source for the alpha-galactose antigen stimulating production of the AAL-reactive IgG is not currently known, given its correlation with the other markers of microbial translocation it is tempting to speculate that it is derived from translocated components of intestinal microorganisms.

We, and others, have observed that the progression of HCV-related liver disease appears to be diminished in injection drug users when compared to persons who acquire infection by transfusion or other routes, even after adjusting for age.^{22, 42} We have also previously reported that liver enzyme values are lower when HCV-infected persons have been injecting in the preceding months.⁴³ Interestingly, we found that active injection drug use was associated with higher levels of protective EndoCAb IgM, which bind LPS. Higher binding antibodies produced in response to chronic LPS stimulation could provide a mechanism for diminished liver disease progression among active injection drug users.

In the absence of an experimental model, we cannot exclude the possibility that microbial translocation is a result of liver disease progression and not a cause. Kupffer cells bind LPS⁴⁴ and other gut-derived microbial products and shunting of portal blood past the liver is a well known consequence of cirrhosis and could explain the elevated levels of plasma LPS.⁴⁵ Indeed, HIV remained associated with liver disease when LPS levels were included in a multivariable model. Admittedly, we were only adjusting for a single LPS determination and consequently underestimating microbial translocation. However, it is most plausible that microbial translocation is both a cause and an effect of liver disease progression and systemic immune activation. As with alcohol-related liver disease, it is possible that abundant microbial

translocation promotes hepatic fibrogenesis, which in turn ultimately increases portal systemic shunting and the abundance of circulating microbial products in a positive feedback loop.

While these data suggest that HIV-related microbial translocation could contribute to liver disease progression among persons with chronic hepatitis C, future studies are needed to dissect how HIV-related microbial translocation causes liver disease progression and to investigate therapeutic interventions.

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Abbreviations

LPS	lipopolysaccharide
LBP	LPS-binding protein
sCD14	soluble CD14
AAL	<i>Aleuria aurantia</i> fucose-binding lectin
EndoCAb IgM	Anti-LPS IgM
TLR4	Toll-like receptor 4
TNFα	tumor necrosis factor alpha

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Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 1E

Figure 1a-e.

Increased microbial translocation and immune activation in HIV-infected subjects with stratified CD4+ lymphocyte counts compared to those without HIV infection. **a.** LPS, **b.** LBP, **c.** sCD14, **d.** AAL, and **e.** EndoCAb IgM was compared in 17 subjects with liver disease and 71 subjects without in the presence and absence of HIV infection. Each marker was measured in stored serum samples. Each point represents the value from one subject's serum. P-values were generated by Wilcoxon Rank-Sum test procedure, and *p<0.05 was considered to be significant.

Figure 2.

HCV seroconversion is not significantly associated with microbial translocation. Thirty-four persons had levels of microbial translocation markers drawn before and after acute HCV seroconversion. LPS levels were not different after seroconversion, as shown. Similar results were found for other microbial translocation markers.

Figure 3.

HIV-related intestinal CD4⁺ lymphocyte depletion and microbial translocation contribute to HCV progression. An integrated model shows that microbial translocation is dependent on HIV-related CD4⁺ lymphocyte depletion. Microbial translocation is critical for the development of immune activation and AIDS, but liver disease develops only in HCV co-infection. Microbial translocation in the host with cirrhosis will lead to less clearance of bacterial products, and increased immune activation.

Table 1Demographic characteristics of study subjects^a.

	Prevalent Liver Disease Cohort		HIV seroconverter Cohort	HCV seroconverter Cohort
	Cirrhosis	Minimal disease		
Total number	17	71	29	34
Mean age (years±SD)	50.5 ± 6.2	41.7 ± 6.0	35.8 ± 6.4	24.4 ± 3.0
Gender, male (%)	10 (58.8)	60 (84.5)	19 (65.5)	21 (61.7)
Race, black (%)	16 (94.1)	69 (97.2)	28 (96.6)	5 (14.7)
HIV (%)	8 (47.1)	20 (28.2)	29 (100)	0 (0) ^b
Proportion injecting illicit drugs (in prior 6 mo)	7 (41.2)	39 (54.9)	26 (89.7)	100%
Alcohol use				
None	11 (64.7)	26 (36.6)	2 (7.1)	not available
>1 drink/day	5 (29.4)	38 (53.5)	16 (57.1)	
7 days/week	1 (5.9)	7 (9.9)	35.7	

^a Cirrhosis was determined by liver biopsy and/or clinical events including esophageal varices, encephalopathy, or ascites.^b Two subjects declined HIV testing and therefore their HIV status was unknown.

Table 2Independent associations of HIV and liver disease with LPS levels in 88 subjects.^a

HIV	Cirrhosis	OR	95% CI	p-value
–	–	1.0	n/a	n/a
+	–	1.21	0.97 – 1.51	0.086
–	+	1.61	1.19 – 2.18	0.0024
+	+	2.07	1.51 – 2.84	<0.0001

^a Cirrhosis was determined by liver biopsy and/or clinical events including esophageal varices, encephalopathy, or ascites.

Table 3
Association of microbial translocation markers and cirrhosis in 88 subjects^a

	OR	95% CI	p-value
Univariate			
LPS ≥ 42 pg/mL ^b	19.0	2.98 – 120.79	0.0018
LBP (highest quartile)	4.39	0.99 – 19.36	0.05
sCD14 (highest quartile) ^b	8.65	1.98 – 37.72	0.0041
AAL ≥ 5 -fold above control ^b	27.77	5.64 – 136.71	<0.0001
EndoCAb IgM (highest quartile)	0.10	0.01 – 0.86	0.036
CD4+ lymphocyte <350/mm ^{3b}	7.02	1.36 – 36.31	0.02
Multivariate^c			
LPS ≥ 42 pg/mL	18.71	2.62 – 133.78	0.0035
CD4+ lymphocyte <350/mm ³	6.29	0.97 – 40.69	0.054

^a LPS-lipopolysaccharide; LBP – LPS binding protein; sCD14 – soluble CD14; EndoCAb IgM antibody to LPS core; AAL- *Aleuria aurantia* lectin. Cirrhosis was determined by liver biopsy and/or clinical events including esophageal varices, encephalopathy, or ascites.

^b Adjusted for age because of an association detected in univariate analysis

^c Shown is a model of cirrhosis, with values adjusted for CD4+ lymphocyte depletion. Similar results were obtained for models of cirrhosis and the other markers (sCD14, LBP, EndoCAb IgM, and AAL) but are not shown. Inclusion of more than one marker in the same model of cirrhosis did not improve the fit significantly.